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BRIEF COMMUNICATION

Complementation of a human adenovirus early region 4 deletion mutant in 293 cells using adenovirus–polylysine–DNA complexes

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The E1 deleted adenoviral vectors are efficient at gene transfer to cells in culture or in animals. However, their use is limited because of an immune-mediated loss of transduced cells. This immune response is believed to result from low-level production of viral antigens from these vectors after gene transfer. The early region 4 (E4) of adenovirus produces a number of proteins that play an important role in adenoviral and host gene regulation during infection of mammalian cells. There is interest in developing E4 deficient adenovirus for gene therapy, especially in the context of developing a combined E1/E4 deleted vector. Towards this goal, a method by which to complement and propagate an E4 deficient adenovirus (dl 1014) in the E1 complementing 293 cell line, using a novel and simple rescue technique, has been developed. Purified adenovirus deficient in E4 gene expression (dl 1014) was conjugated to expression plasmids containing the E4-open reading frame 6 gene or complete E4 region to produce adenovirus-polylysine-DNA complexes that were used to transfect 293 cells. The derived virus obtained from this transfection did not replicate on 293 cells but did replicate on W162 cells (E4+) confirming that the virus was indeed deleted for E4. Viral yield was high ranging from 3×10^{7} to 9×10^{8} plaque forming units per 10^{6} 293 cells. This method has general application to the production of new adenoviral mutants that will be useful for developing second generation adenoviral vectors.

Keywords: adenovirus dl 1014; E4; polylysine complexes; gene therapy

Early region 4 (E4) of human adenoviruses encodes seven proteins and occupies about 3000 base pairs at the right end of the viral genome. Analysis of adenovirus mutants has shown that E4 proteins are necessary for normal progression of virus infection into the late phase of the infectious cycle. E4 defective adenovirus mutants have a complex phenotype which includes defects in accumulation of late RNA, efficiency of late protein synthesis, viral DNA replication and a failure to shut-off host cell protein synthesis.[1–8] The products of two E4 open reading frames (ORFs 3 and 6) have redundant functions in late viral gene expression and the expression of either one seems to be sufficient to establish an essentially wild-type virus infection.[5,6,7–11] The E4-ORF6 protein also forms a complex with the EIB-55K protein during a lytic infection and this complex functions to block cytoplasmic accumulation of host mRNA and facilitate transport of late viral mRNAs.[3,6,12] The third E4 protein which has been characterized is E4-ORF6/7 which is involved in augmenting transcription of early region E2 by facilitating a cooperative binding of the transcription factor E2F to the E2 promoter.[13–17]

Mutants that have deletions in both E4 ORF3 and E4 ORF6 are defective for growth on normal adenoviral host cells but can be propagated in cells expressing the E4 region, the W162 cell line.[18] For host E4 mutants, defects in DNA replication are not observed at high multiplicities of infection[8,10,19] and E4 products are not absolutely required for DNA replication. The E4 mutant Ad5 dl 1014 carries two deletions that together destroy all E4 ORFs except ORF4.[8] Ad5 dl 1014 is highly defective in viral DNA replication because the product of ORF4 is responsible for the inhibition of viral DNA replication in Ad5 dl 1014 infected cells. ORF3 and ORF6 products can antagonize the effect of ORF4 and therefore, viruses that express either ORF3 or ORF6 are not subject to inhibition by ORF4.[8] As predicted, Ad5 dl 1014 can be propagated in the W162 cell line but not in the HeLa and 293 (E1+) cell lines.

Currently, E1 deleted replication defective recombinant adenoviral vectors have received much attention because of their ability to transduce a large proportion of cells in a number of different organs in animal models (see review by Kay and Woo, 1994)[20]. Unfortunately, the production of small amounts of viral antigens limits the use of these vectors for gene

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therapy because of the associated immune response directed against transduced cells *in vivo*.[21] Thus, it is highly desirable to produce recombinant vectors with deletions in more than one genetic region. Because of the regulatory role of E4 gene products, it represents a prime area of the viral genome to be considered for deletion in gene therapy vectors. To date, it has not been possible to produce a stable cell line that can complement both E1 and E4 functions[18] because many adenoviral gene products are toxic to cells when constitutively produced.

In theory it should be possible to complement adenoviral mutants by transient expression of the appropriate adenoviral gene products, however, standard transient transfection methods (ie calcium phosphate precipitation, lipofection and electroporation) are difficult to optimize and in general produce only modest levels of gene product. This is due in part to inefficient DNA uptake and/or delivery to the nucleus. A promising method has been developed recently by which gene expression from plasmid DNAs can be increased by three orders of magnitude in a variety of different cell types.[22-24] This method uses molecular conjugates containing plasmid DNA complexed to an adenoviral particle. The plasmid DNA is delivered at high efficiencies into the cell and nucleus via the adenoviral receptor. We have exploited this method by constructing E4-ORF6 expression plasmids and using these to produce adenovirus dl 1014–polysine–DNA complexes that complement the E4 gene region when transferred into 293 cells. The result was the production of high-titer replication deficient E4 mutant Ad5 dl 1014 virus in 293 cells. This method may have general use for complementation of adenoviral mutants for biologic studies as well as for the generation of new adenoviral vectors for gene therapy.

The *Eco*RI fragment that contains the complete E4 region (83.3 to 100 map units) from adenovirus type 5 that was originally derived from the p*Eco*RIBAd5 plasmid[25] was cloned into the *Eco*RI site of pBluescript SK, (Stratagene, La Jolla, CA, USA) to produce pBSRIB (gift of J Boyer and G Ketner, Johns-Hopkins University). The ORF6 coding sequence was amplified by the polymerase chain reaction (PCR) from the pBSRIB plasmid and cloned into two expression plasmids (Figure 1). pLNCX contains a recombinant retroviral vector,[26] and pCEP4 (Invitrogen, San Diego, CA, USA) is an expression plasmid. Both plasmids express the ORF6 from the cytomegalovirus (CMV) promoter. The pBSRIB expresses the E4 gene products from the endogenous viral promoter. The Ad5 dl 1014 E4 deletion mutant was selected for complementation studies because of the presence of an intact E4-ORF4 sequence which renders the virus completely replication defective in all cell lines except W162 cells.[8] Ad5 dl 1014 adenovirus–polylysine–DNA conjugates containing different plasmids were transfected into 293 cells (Figure 1). After 4 days, the 293 cells transfected with dl 1014 adenovirus conjugated with the pCEP/ORF6, pLNCX/ORF6 or pBSR1B plasmids had complete

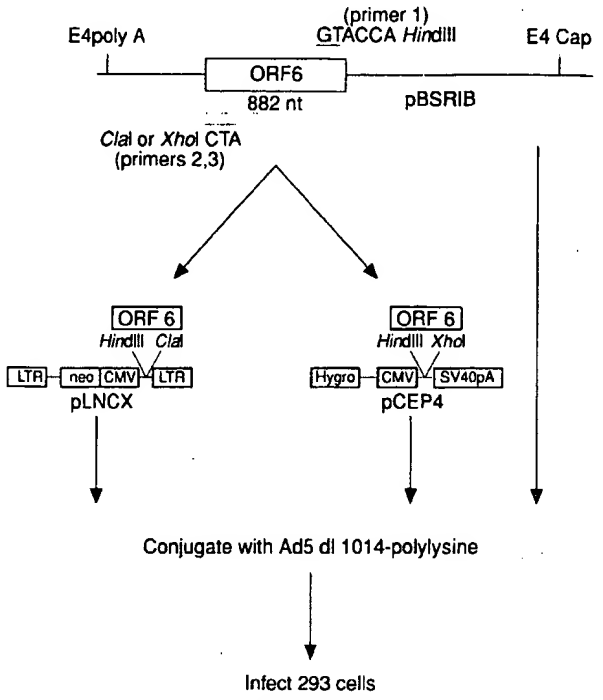

Figure 1 Construction of the E4-ORF6 expression plasmids and the production of Ad5 dl 1014 adenovirus–polylysine–DNA conjugates. Primers containing specific restriction sites and the corresponding 5′ and 3′ ends of the ORF6 sequence were used to PCR amplify the gene from the pBSR1B plasmid.[28] The double stranded PCR product was cloned into the pLNCX plasmid and the pCEP4 plasmid (Invitrogen, San Diego, CA, USA).[26] The figure shows a portion of each of the plasmids. Ad5 dl 1014 viral stocks were prepared on W162 cells and purified by double cesium chloride banding.[31] 1 × 10[10] dl 1014 adenovirus–polylysine particles in 100 µl of buffer were mixed with 6 µg of plasmid DNA and prepared as previously described for transfection.[29,30] The molecular conjugates were added to 1 × 10[6] 293 cells in 60-mm dishes and cultured for 4 days in 5 ml of media containing High glucose DMEM (Hyclone, Logan, UT, USA) and 10% fetal calf serum. hygro, hygromycin resistance gene; neo, neomycin resistance gene; LTR, long terminal repeat

cytopathic effects (CPE) whereas the cells treated with the conjugates containing the pCMV-βgal plasmid or no plasmid did not have CPE.[27] The quantity of virus produced from the molecular conjugates after transfection in the cultured 293 cells, was determined by plaque titering on W162 cells (Table 1). High titers of adenovirus ranging from 3 × 10[7] to 9 × 10[8] plaque forming units (p.f.u.) were recovered from all the 293

Table 1 Complementation of Ad5 dl 1014 adenovirus with E4 expression plasmids

Adenovirus–DNA conjugate	*Virus recovery*	
	Experiment 1	*Experiment 2*
dl 1014	<2 × 10[2]	<2 × 10[2]
dl 1014 + pCMVβgal	<2 × 10[2]	<2 × 10[2]
dl 1014 + pLNCX-ORF6	4 × 10[7]	3 × 10[7]
dl 1014 + pCEP-ORF6	9 × 10[8]	8 × 10[8]
dl 1014 + pBSR1B	8 × 10[8]	ND

293 Cells transfected with the molecular conjugates and culture media were freeze-thawed three times and the cell debris removed by centrifugation. The supernatants were titered as described on W162 cells.[8] The number of p.f.u./ml was calculated and then multiplied by volume of viral supernatant to give the final virus recovery. ND, not determined

cells transduced with the plasmids that contained the ORF6 gene but no detectable virus was obtained from the cells transduced with Ad5 dl 1014 adenovirus alone or with the control pCMV-βgal plasmid. There were no major differences in viral yield between the experiments using the expression plasmids that only contained the ORF6 gene or a plasmid that contained the complete E4 region (pBSR1B).

It is possible that ORF6 plasmid DNA and the Ad5 dl 1014 genome underwent recombination to produce virus that contains E4-ORF6 and thus, would behave as a wild-type virus. If the recovered virus does contain the E4-ORF6 gene it will replicate on 293 cells. Thus, in order to evaluate the recovered virus for replication on 293 cells, about 1/20 of the viral stocks (2×10^6 to 4×10^7 p.f.u.) obtained from the above experiments were used to reinfect 2×10^5 293 cells and W162 cells. As little as one wild-type p.f.u. in 1×10^5 viral particles leads to CPE in 293 cells within 1 week of culture. None of the viral stocks produced CPE on 293 cells after more than 1 week whereas CPE was observed within several days on W162 cells. This confirmed the absence of replication competent E4-containing virus and demonstrates that the complementation of the Ad 5 dl 1014 mutation occurred as a result of gene expression from the E4-ORF6-containing plasmids.

Recombinant adenoviral vectors that are deficient in E1 regions have severe limitations that include the low-level production of viral antigens that cause inflammation and immunologic responses against the transduced cells, and deletion of E4 function may be advantageous for the production of second generation adenoviral vectors. Until a cell line that can stably produce both E1 and E4 gene products can be produced, the methods outlined here may be useful for generating E1/E4 deficient vectors for animal experimentation. In general, the method described here can be used for constructing a number of different adenoviral mutants.

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